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Award Number: DAMD17-01-1-0252

TITLE: An Epithelial-Derived, Integral Membrane, Kunitz-Type
Serine Protease Inhibitor in Breast Cancer

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REPORT DATE: August 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20030211 186

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE

August 2002

3. REPORT TYPE AND DATES COVERED

Annual Summary (1 Aug 01 - 31 Jul 02)

4. TITLE AND SUBTITLE

An Epithelial-Derived, Integral Membrane, Kunitz-Type Serine Protease Inhibitor in Breast Cancer

5. FUNDING NUMBERS

DAMD17-01-1-0252

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract (Maximum 200 Words) *(abstract should contain no proprietary or confidential information)*

In the current research plan, we proposed to study the anti-tumor and anti-protease activity of a membrane-bound Kunitz-type serine protease inhibitor (KSPI; also known as HAI-1). In order to determine the effects of HAI-1 expression on breast cancer cell biology, we have transfected breast cancer cells MDA MB 435 cells with HAI-1 and selected 6 stable clones. In the first year, we have characterized these HAI-1 overexpressing breast cancer cells in vitro and in vivo, comparing to their parental cells and neo control. HAI-1 expression profoundly reduced the proliferation and *in vivo* tumor growth of MDA MB-435 cells. Additionally, HAI-1 expression altered the cellular morphology of this cell line, but had no effect on the cell motility. These results indicate that HAI-1 expression may inhibit tumor progression by modulating tumor growth, in a manner that is dependent upon the serine proteases, their substrates, and substrate effector molecules expressed by individual breast cancer cells.

14. SUBJECT TERMS

anti-tumor, protease, protease inhibitor, cancer invasion, HGF activation, pro-uPA activation, FGF, breast cancer

15. NUMBER OF PAGES

9

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT

Unclassified

18. SECURITY CLASSIFICATION
OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION
OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)

Prescribed by ANSI Std. Z39-18
298-102

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Introduction:

The death of women with breast carcinoma mainly results from metastasis. Metastatic breast cancer cells must escape from a primary tumor and migrate through anatomical barriers in order to gain access to the blood or lymphatic system and establish at a new site in the body. Cellular motility and degradation of extracellular matrix (ECM) are two of the major events in breast cancer metastasis and can be promoted by stromal-derived, ECM-degrading protease systems, such as the urokinase type plasminogen activator (uPA) system and by motility factors, such as hepatocyte growth factor (HGF)/scatter factor (SF). In order to understand how breast cancer cells regulate both stromal-derived, ECM degradation and cellular motility for metastasis, we have discovered and characterized in human breast cancer a new epithelial-derived, type 2 integral membrane, serine protease, matriptase, and its cognate inhibitor, a Kunitz-type serine protease (KSPI), a type 1 integral membrane protein which was initially identified as an inhibitor of hepatocyte growth factor activator and named as HAI-1 (1-3). Both matriptase and HAI-1 have been implicated in the regulation of ECM-degradation of cellular motility (4). In the current research plan, we proposed to study the anti-tumor and anti-protease activity of this membrane-bound Kunitz inhibitor.

Body:

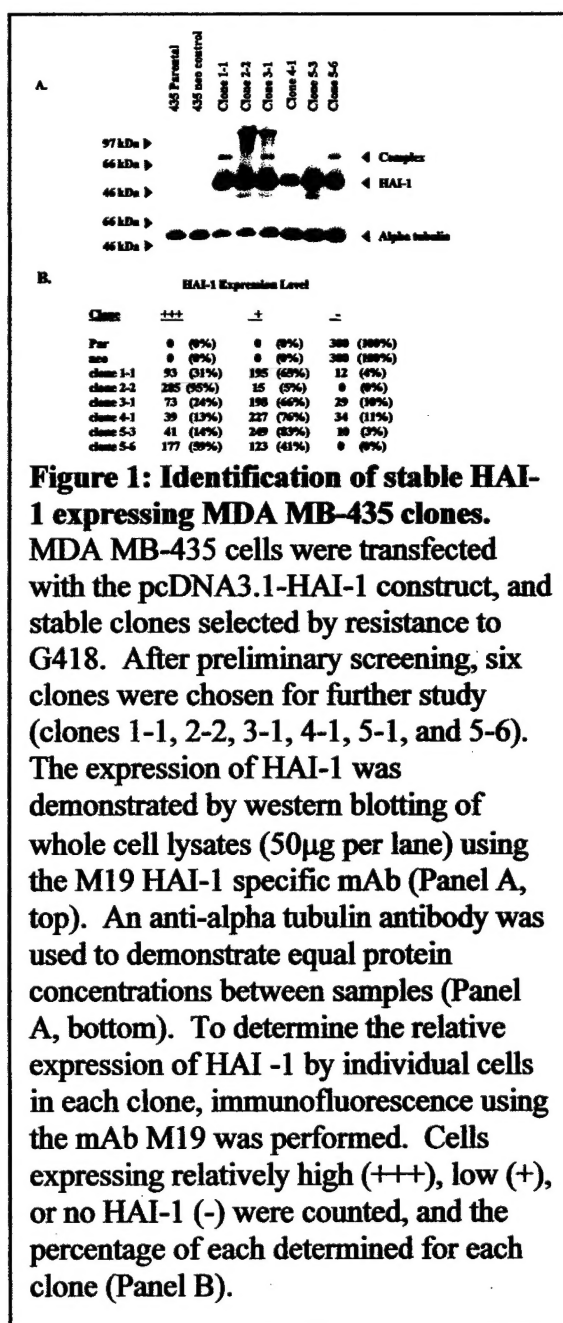
During the August 01-July 02 period we had addressed Aim 1 and a portion of Aim 2 of the original proposal:

Year 1: In the first year, we will carry out the experiments regarding anti-tumor activity of KSPI (also known as HAI-1) (Aim 1). These include transfection of KSPI into α 18 and MDA MB-435 breast cancer cells, selection of stable clones, evaluation of both transfectants in terms of their *in vitro* growth and invasion and their *in vivo* metastatic potential. We will also begin to generate KSPI mutants by site-directed mutagenesis (Aim 2)

Generation of stable HAI-1 expressing MDA MB-435 cell lines.

As the first step toward approach Aim1, we have first transfected MDA MB 435 breast cancer cells with the Kunitz inhibitor HAI-1 (KSPI). These colonies were analyzed by western blot for HAI-1 expression (Figure 1A), and six of the strongest HAI-1-positive clones (clones 1-1, 2-2, 3-1, 4-1, 5-3, and 5-6), and a single *neo* control clone, were used for further study. In addition to observing the 66kDa membrane-bound form of HAI-1, a higher molecular weight band was also observed. This band was eliminated by the boiling of samples prior to SDS-PAGE (data not shown), indicating that it is probably a complex between HAI-1 and another unidentified protein. In addition to its presence in whole cell lysates, HAI-1 was also detected in the cell-conditioned media from HAI-1 expressing clones in an independent experiment (Data not shown). Immunofluorescence analysis of the HAI-1 positive clones revealed that each of the clones was heterogeneous with regard to HAI-1 protein expression, with a variety of cells displaying high, low, or no HAI-1 expression (Data not shown). Counting of randomly selected fields revealed that clones 1-1, 2-2, and 5-6 had a high percentage of strongly HAI-1 positive cells, whereas clones 3-1, 4-1, and 5-3 contained a proportion of weakly staining, or negative, HAI-1 cells in addition to strongly positive cells (Figure 1B).

Growth of HAI-1 over-expressing MDA MB-435 breast cancer cells on tissue culture plastic and in soft agar. The



growth rate of HAI-1 expressing MDA MB-435 clones was assessed by crystal violet staining of cells grown in 96-well plates. All HAI-1 expressing clones grew at a slower rate than the parental and *neo*-transfected control cell lines (Figure 2). Clones with a high percentage of HAI-1 positive cells (clones 1-1, 2-2, and 5-6) grew very poorly, while clones expressing a lower percentage of strongly HAI-1 positive cells (clones 3-1, 4-1, and 5-3) grew at an intermediate rate. Clones with a higher percentage of HAI-1 positive cells tended to lose HAI-1 expression upon multiple passages, possibly due to silencing of the CMV promoter driving HAI-1 expression in these cells (data not shown). The growth rate of these clones rose as the expression of HAI-1 fell (data not shown), reinforcing that HAI-1 expression, and not clonal variability, was responsible for the observed growth suppression of HAI-1 expressing MDA MB-435 clones.

The growth inhibition of HAI-1 expressing MDA MB-435 clones is the result of slowed cell cycling, not apoptosis. To assess whether the growth inhibition of HAI-1 expressing MDA MB-435 clones is due to either slowed proliferation or the induction of apoptosis, cells were analyzed using flow cytometry-based cell cycle analysis (Vindelov method) and by Hoechst dye 33258 staining of cell nuclei, respectively. Cell cycle analysis of HAI-1 clones revealed that HAI-1 expression reduced the S-phase fraction of the clones (Data not shown). However, no change was observed in the number of cells with condensed nuclei, indicating no difference in the apoptotic fraction (Data not shown). Thus, the growth inhibitory effect of HAI-1 expression in MDA MB-435 cells is due to slowed cellular proliferation, not apoptosis.

The motility of HAI-1 expressing MDA MB-435 clones is unaffected by HAI-1 expression. For the assessment of cell motility, the phagokinetic gold motility assay was used. In this assay, cells were plated onto glass coverslips that had been coated with colloidal gold and fibronectin. When cells travel on the coverslips, they phagocytose colloidal gold and leave behind visible tracks. HAI-1 expression in the MDA MB-435 clones did not alter the overall clearance of colloidal gold in this assay (Figure 3). However, it is interesting to note that MDA MB-435 clones that express HAI-1 cleared more colloidal gold in the motility assay on a *per cell* basis (number of cells can be estimated by counting DAPI stained nuclei). The parental MDA MB-435 cells spend more time in mitosis, and are more crowded after cell division due to their increased growth rate. They may therefore encounter one another more frequently in random motility compared to the slower-growing HAI-1 expressing clones. Thus, it

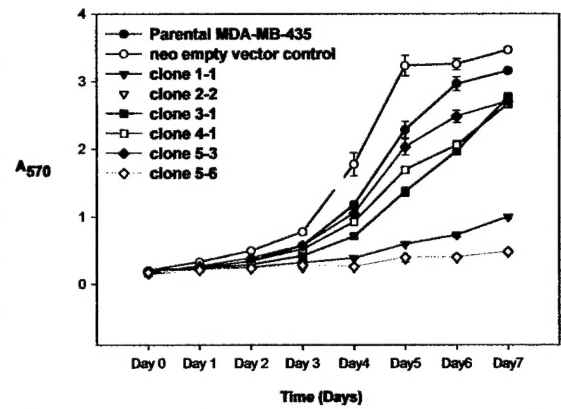


Figure 2: MDA MB-435 clones expressing HAI-1 grow at a slower rate than either parental or neo-transfected MDA MB-435 cells. Cell proliferation of HAI-1 expressing MDA MB-435 clones was assessed relative to parental and *neo*-transfected cells by the crystal violet growth assay. All HAI-1 expressing clones grew at a slower rate than either parental or neo-transfected cells. The effect of HAI-1 expression on cell growth was proportional to the percentage of cells expressing high levels of HAI-1 within each clone. For example, clones 1-1, 2-2, and 5-6 grew very poorly, whereas clones 3-1, 4-1, and 5-3 grew at intermediate rates.

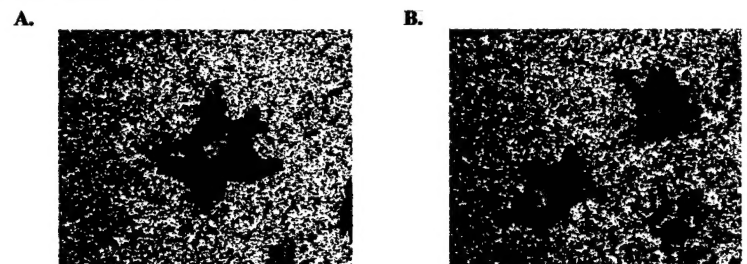


Figure 3: The overall motility of MDA MB-435 cells is not affected by HAI-1 expression. The phagokinetic gold motility assay was used to assess the effects of HAI-1 expression on the motility of clone 1-1 and 2-2 cells, as compared to that of parental and *neo*-transfected cells. In this assay, cells were plated onto colloidal gold covered microscope coverglasses and cultured for 48 hours. Clone 1-1 (Panel A) and 2-2 cells did not clear more gold overall in this assay than either parental (Panel B) or *neo*-transfected cells. However, the HAI-1 expressing cells did leave greater tracks on a *per-cell* basis, after estimation of cell number by counting DAPI-stained nuclei. The effects of cell crowding, or cell division, in parental and *neo*-transfected cells may explain this phenomenon.

is unlikely that this increase in colloidal gold clearance *per cell* represents a true increase in cell motility.

MMP activity in cell conditioned media is unaffected by HAI-1 expression in HAI-1 expressing MDA MB-435 cells. Serine proteases, such as uPA and plasmin, have been shown to activate members of the matrix metalloproteinase family. Therefore, we assessed whether the level of MMP-2 and MMP-9 activity in the cell conditioned media changed with HAI-1 expression, and whether this may be associated with the change in growth seen in HAI-1 expressing MDA MB-435 cells. MDA MB-435 parental, *neo*-transfected, and clone 1-1 and 2-2 cells were grown for 24 hours in serum-containing media, after which time it was replaced by media containing only ITS, and cells were cultured for an additional 48 hours. Cell-conditioned media was collected and concentrated approximately 30-fold. After careful measurement of the sample volume, a normalized equivalent of conditioned media was resolved by SDS-PAGE for western blot or gelatin zymography. Normalization was achieved by using a volume that was directly proportional to the protein concentration of whole cell lysates made from cells remaining on the culture dish (to correct for differences in cell growth). Examination of western blotting for HAI-1 revealed that the HAI-1 expressing MDA MB-435 clones shed HAI-1 into the conditioned media (Data not shown). Gelatin zymography of the same conditioned media showed that MMP-2 and MMP-9 levels did not significantly differ among most of the HAI-1 expressing MDA MB-435 and parental or *neo* cells. One exception was a substantially higher MMP-9 activity, and a slightly increased in MMP-2 activity, seen for clone 1-1 that apparently was the result of clonal variability (Data not shown).

Chemoinvasion of HAI-1 expressing MDA MB-435 clones in the Matrigel invasion assay. The ability of HAI-1 expressing MDA MB-435 clones to invade through Matrigel was examined in the Matrigel chemoinvasion assay. After plating of cells in Matrigel invasion chambers, cells were incubated for an additional 24 hours to allow cells to migrate through a Matrigel-coated membrane towards media conditioned by cultured human breast fibroblasts. The migration requires that cells degrade the Matrigel and crawl through 8 μ m pores to the opposite side of the membrane. After migration, cells can be removed from the plating side of the membrane with a cotton applicator, and cells that have migrated to the opposite side can be visualized by staining with crystal violet. Examination of cells stained with crystal violet showed little difference in the number of cells that had migrated when comparing parental cells, *neo*-transfected, and HAI-1 expressing clones (Data not shown). The assay showed considerable intra- and interassay variability, but repeated experiments supported the premise that HAI-1 over-expression did not alter the motility of MDA MB-435 cells.

Growth of HAI-1 expressing MDA MB-435 clones as tumor xenografts in the nude mouse. HAI-1 expressing MDA MB-435 clones 1-1 and 2-2, parental, and *neo*-transfected control cells were grown as tumor xenografts in NCr nu-/nu- (athymic) nude mice. Each cohort contained ten mice, and each mouse was injected with 5X10⁵ tumor cells subcutaneously over the mammary fat pad between nipples 3 and 4. The tumors from each cohort were allowed to grow to a mean volume of approximately 1000mm³ each, at which time the cohort was sacrificed as a whole. This was done because the metastatic potential of tumor cells is widely believed to be proportional to tumor size, and so the mean tumor size was kept constant at the time of sacrifice, regardless of tumor growth rate.

Tumors derived from the HAI-1 expressing clones 1-1 and 2-2 grew significantly ($p < 0.05$) slower than either parental or

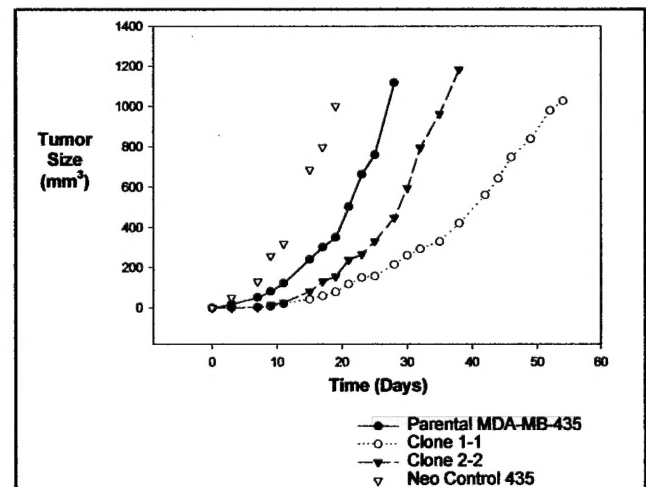


Figure 4: The growth of clone 1-1 and 2-2 tumor xenografts is significantly inhibited relative to the growth of parental and *neo*-transfected tumor xenografts. Five hundred thousand tumor cells from parental, *neo*-transfected, and clones 1-1 and 2-2 were implanted subcutaneously on each side of a cohort of ten NCr nu-/nu- mice (20 tumors total per cohort) between nipples 3 and 4. After implantation, tumor volume was measured approximately twice weekly. A plot of mean tumor volume for each of the twenty tumors was graphed versus time after tumor implantation. The graph shows that tumor xenografts from clones 1-1 and 2-2 grow at a significantly reduced rate ($p < 0.05$) as compared to tumor xenografts from parental or *neo*-transfected cells.

Comparisons	Estimated difference (mm ³)	95% CI for the difference (mm ³)	Adjusted P value
Neo/Par	791.14 (387.66 [*])	-252.93, 1835.2	0.19
Neo/clone 2-2	1257.7 (379.62)	235.29, 2280.12	0.01
Neo/clone 1-1	1518.23(379.35)	496.57, 2539.9	0.0016
Par/clone 2-2	466.57 (161.8)	30.8, 902.33	0.03
Par/clone 1-1	727.1 (161.15)	293.09, 1161.1	0.0004
clone 1-1/2-2	-260.53 (140.72)	-639.51, 118.45	0.27

Table 1: Statistical comparison of tumor volume means between cohorts of MDA MB-435 tumor-bearing mice. Comparisons were made between tumor volume means between individual cohorts of mice bearing parental MDA MB-435 (Par), neo-transfected control (Neo), clone 1-1, or clone 2-2 tumors by the Tukey method. The least square means of tumor volume of the neo-transfected (1704.57) and parental (913.43) groups are significantly larger than those of the clone 1-1 (186.34) and clone 2-2 (446.87) groups at 3% (adjusted) or less significance level, and the 95% confidence intervals for the differences are substantially above zero.

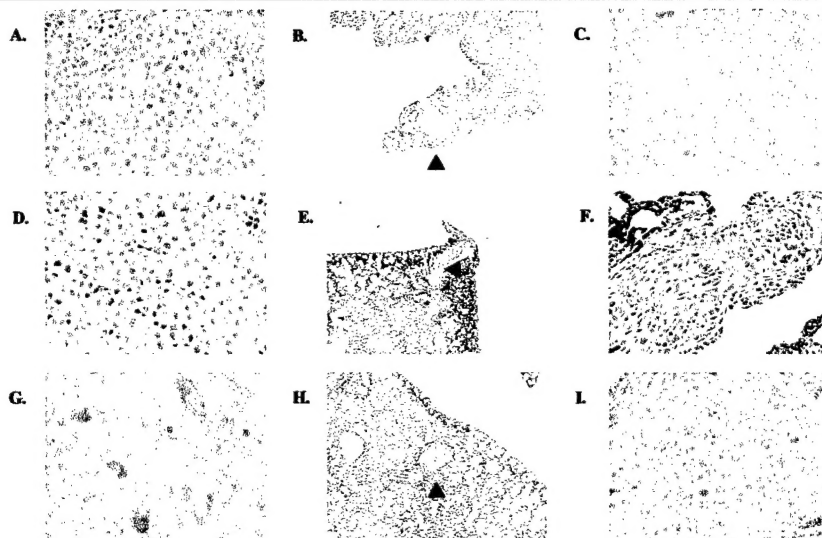


Figure 5: Confirmation of HAI-1 expression in clone 1-1 and 2-2 primary tumor xenografts and metastases by anti-HAI-1 immunohistochemistry. To determine if HAI-1 was expressed by tumor xenografts, and corresponding lung metastases, at the time that they were removed from mice, HAI-1 specific immunohistochemistry was performed using the M58 mAb on paraffin-embedded sections. Staining of primary tumors from mice harboring parental (Panel A, primary tumor, 100X) or *neo*-transfected control (Panel D, primary tumor, 100X) tumors did not show evidence of HAI-1 staining as expected. In addition, HAI-1 staining of lung metastases (arrowhead) in mice harboring parental (Panel B, lung metastasis, 25X and Panel C, lung metastasis, 100X) or *neo*-transfected control (Panel E, lung metastasis, 25X and Panel F, lung metastasis, 100X) tumors also did not show evidence of HAI-1 staining, also as expected. Staining of primary tumors from mice harboring clone 2-2 tumors (Panel G, primary tumor, and J, primary tumor, 100X) or clone 1-1 tumors (Panel M, primary tumor, 100X), however, did show evidence of HAI-1 staining in most primary tumors. Staining was absent, however, in the lung metastases (arrowhead) from clone 2-2 tumor bearing mice (Panel H, lung metastasis, 25X and Panel I, lung metastasis, 100X; Panel K, lung metastasis, 25X, and Panel L, lung metastasis, 100X), and from one clone 1-1 tumor bearing mouse, although another clone 1-1 tumor bearing mouse did show evidence of HAI-1 staining in lung metastasis (Panel N, lung metastasis, 100X, and Panel O, lung metastasis, 25X). Some staining of mouse lung tissues is seen in sections of mouse lung since the secondary antibody used in the M58 mAb IHC likely cross-reacts with endogenous mouse IgG or other mouse proteins.

neo-transfected tumors (Figure 4). The statistical comparisons between mean tumor volumes for each individual cohort are presented in Table 1. The expression of HAI-1 in the implanted MDA MB-435 tumors was confirmed by both M19 mAb western blotting (Data not shown) and immunohistochemistry (Figure 5) using the HAI-1 specific antibody M58 (5-6). The expression of HAI-1 was seen in only a subset of tumor cells (about 10-40%) in tumors derived from clone 1-1 and 2-2; intra and inter-tumor HAI-1 expression was variable. Some tumors showed few HAI-1 positive cells. These observations were consistent with *in vitro* experiments that showed that HAI-1 expression was reduced after continued passage of these cells in culture (data not shown), and suggest that HAI-1 expression may be reduced *in vivo* over time as well. To determine if HAI-1 expression by the tumor cells affected the vascularization of tumors, sections were stained using a PECAM-specific monoclonal antibody, and tumor vasculature was examined (Data not shown). No difference in vascularization was seen between HAI-1 expressing tumors and parental or *neo*-transfected control tumors.

The ability of tumor cells to metastasize to the lungs, kidneys, and liver of mice was assessed by examining H&E stained sections for the presence of human tumor cells within these organs. Metastatic disease was only observed in the lungs of mice, present predominately as micro-metastatic foci. No significant difference in the number, or general size, of metastatic lesions was observed between mice harboring HAI-1 expressing MDA MB-435 tumors and parental or *neo*-transfected tumors (Data not shown). Interestingly, however, among metastatic foci that were found, HAI-1 immunohistochemical staining was observed in metastatic disease in only a single mouse harboring HAI-1-expressing tumors out of five that had metastatic foci that could be evaluated (Data not shown).

Key research accomplishments:

- We have generated stable HAI-1 expressing MDA MB-435 cell lines
- We have characterized the morphology of MDA MB-435 HAI-1 clones grown on tissue culture plastic and in Matrigel matrix
- The growth of HAI-1 over-expressing MDA MB-435 breast cancer cells on tissue culture plastic and in soft agar has been determined.
- The growth inhibition of HAI-1 expressing MDA MB-435 clones is the result of slowed cell cycling, not apoptosis.
- The motility of HAI-1 expressing MDA MB-435 clones is unaffected by HAI-1 expression.
- MMP activity in cell conditioned media is unaffected by HAI-1 expression in HAI-1 expressing MDA MB-435 cells.
- We have tested the chemoinvasion of HAI-1 expressing MDA MB-435 clones in the Matrigel invasion assay.
- We have finished the growth of HAI-1 expressing MDA MB-435 clones as tumor xenografts in the nude mouse.

Reportable outcomes:

1. Oberst, M.D., Anders, J., Benaud, C., Lin, C.-Y., Dickson, R.D., and Johnson, M.D., (2001) Over-expression of the hepatocyte growth factor inhibitor (HAI-1) in human breast cancer cells: Effects on cell growth, morphology, and motility. 2nd General Meeting of International Protease Society, Muenchen, German.
2. Oberst, M.D., Anders, J., Dickson, R.D., Johnson, M.D., and Lin, C.-Y., (2002) Over-expression of the hepatocyte growth factor inhibitor (HAI-1) affects the growth in human breast cancer cells. Era of Hope 2002, DOD Breast Cancer Research Program Meeting, Orlando, Florida
3. Oberst, M.D., Anders, J., Dickson, R.D., Lin, C.-Y., and Johnson, M.D (2002) Over-expression of the hepatocyte growth factor inhibitor (HAI-1) affects the growth in human breast cancer cells. Manuscript in preparation and will be submitted to Clinial & Experimental Metastasis

Conclusion:

In order to determine the effects of HAI-1 expression on breast cancer cell biology, HAI-1 was over-expressed in breast cancer cell line, MDA MB-435 in a stable fashion. HAI-1 expression profoundly reduced the proliferation and *in vivo* tumor growth of MDA MB-435 cells. Additionally, HAI-1 expression altered the cellular morphology of both cell lines, but had no effect on the cell motility. These results indicate that HAI-1 expression may inhibit tumor progression by modulating tumor growth, in a manner that is dependent upon the serine proteases, their substrates, and substrate effector molecules expressed by individual breast cancer cells.

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Appendices: None